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Regulation of the Production of Hemicellulolytic and Cellulolytic Enzymes by a *Streptomyces* sp. Growing on Lignocellulose

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A *Streptomyces* sp. isolated from compost degraded the hemicellulose fraction of straw efficiently but apparently not native cellulose. Ball-milled straw induced endoglucanase, β-glucosidase, β-xylanase and β-xylosidase. Carboxymethylcellulose, cellotetraose and cellotriose induced cellulolytic enzymes specifically whereas cellobiose acted as inducer for β-glucosidase only. Cellotriose and cellotetraose induced β-glucosidase, but only partially induced endoglucanase. Hemicellulose (in the form of xylan) and xylobiose induced only β-xylanase and β-xylosidase. Kraft lignin and syringic acid induced β-xylanase and endoglucanase but not the other enzymes. 3,4-Dimethoxycinnamic acid slightly induced β-xylanase whereas 3,5-dimethoxy-4-hydroxycinnamic acid specifically induced endoglucanase. Neither veratric acid nor vanillic and ferulic acids induced any of the cellulolytic or hemicellulolytic enzymes. Enzyme production was subject to a form of carbon catabolite repression. Endoglucanase and β-xylanase were excreted into the culture medium. Four protein components, one acidic (pI 5·2) and three basic (pI 8·15, 8·45 and 8·65) exhibited β-xylanase activity. Two acidic components (pI 3·55 and 3·75) displayed endoglucanase activity.

INTRODUCTION

Actinomycetes play a considerable role in recycling nutrients in natural and man-made environments. For example, they are thought to be involved in the primary degradation of organic matter in compost and related materials (Goodfellow & Williams, 1983). Lignocellulose-degrading actinomycetes have been isolated from compost but little is known about their growth pattern and control of lignocellulolytic enzyme synthesis (McCarthy, 1987). Previous work has shown the potential of actinomycetes isolated from agricultural and garden compost to degrade cellulose (Godden & Penninckx, 1984; Van Zyl, 1985). Detailed knowledge of the physiological requirements of enzyme production is a prerequisite for efficient future exploitation of actinomycetes in lignocellulose bioconversion.

Here we report a physiological study of a strain of *Streptomyces* isolated from cattle manure compost (Godden & Penninckx, 1984). This strain is an efficient hemicellulose-degrading organism. It also produces a cellulolytic enzyme and was thus chosen as a model for evaluating factors governing the synthesis of both cellulose- and hemicellulose-degrading enzymes. We also used ball-milled wheat straw as a realistic lignocellulose substrate which might be used in bioconversion.

METHODS

Organism and culture conditions. The *Streptomyces* sp., strain EC1, was isolated from cattle manure compost (Godden & Penninckx, 1984). It is mesophilic. This strain was selected for study on the basis of its ability, in an initial screening, to produce straw-saccharifying enzymes (Ball & McCarthy, 1988). It was maintained either

Abbreviations: CMC, carboxymethylcellulose; DNS, dinitrosalicylic acid; RBR, remazol brilliant blue R.

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frozen at −80 °C in 80% (w/v) glycerol or at 4 °C as one-week-old slant cultures on Difco actinomycetes isolation agar. Spore suspensions prepared from such slants (Hopwood *et al.*, 1985) were resuspended in 0·9% NaCl and used for inoculation of the experimental media. The basal medium contained 2 g $(NH_4)_2SO_4$, 0·2 g $MgCl_2.6H_2O$, 11 mg $CaCl_2$, 2 g K_2HPO_4 and 0·6 g KH_2PO_4 per litre of distilled water, final pH 7·5; it was sterilized for 15 min at 121 °C. Before adding the carbon source, each litre of cooled medium (50 °C) was supplemented with 20 ml of a vitamin solution (Malfait *et al.*, 1984). The carbon source was added as indicated in the text. The substrates were sterilized by filtration except xylan and straw, which were sterilized for 15 min at 121 °C.

In order to ensure aerobic conditions, the volume of the medium was restricted to 1/5 of the total volume of each flask. A stainless steel spring was inserted into the flask to favour dispersed growth in an orbital incubation shaker (154 strokes min^{-1}). Cultures were grown at 37 °C.

In the growth experiments, about 10 mg dry weight of pregerminated spores in 30 ml was inoculated into 300 ml of medium containing the desired carbon source. After 48 h of growth, this preculture was added to 3 l of fresh medium containing the same carbon source. Samples (100 ml culture medium) were taken and analysed as indicated in the text.

In induction experiments, 20 ml of spore suspension (10 mg dry weight) was first inoculated into 200 ml of a culture medium containing 0·2% glycerol as the carbon source. After 48 h of growth, the cells were harvested by filtration on a Millipore 0·45 μm membrane under sterile conditions, and inoculated into 400 ml of the induction medium being tested.

Cellotriose and cellotetraose were a gift from Dr M. Clayssens, University of Ghent, Belgium. Kraft lignin was obtained from Dr A. Ball, University of Liverpool, UK.

Enzyme determination. The sample of culture medium to be analysed was first centrifuged at 27000 ***g*** for 10 min. The pellet was resuspended in 3 ml 10 mM-potassium phosphate pH 7·0 and sonicated with cooling at 4 °C for 10 min with a Vibra Cell VC 500 ultrasonic processor (Sonics Materials). The suspension obtained was clarified by centrifugation at 27000 ***g*** for 10 min and the supernatant (i.e. crude extract of the bacteria) used for the assay of β-glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37).

β-Xylanase (EC 3.2.1.8) and endoglucanase (EC 3.2.1.4) were assayed in the supernatant from the first centrifugation (i.e. culture medium).

In growth experiments where sugars could interfere with the enzyme assays, these were first removed by chromatography on a calibrated Biogel P2 column. The conditions of enzyme assay (pH, temperature, substrate concentration, incubation time) were optimized in preliminary experiments. Rate of product release was linear with time for up to 2 h with appropriately diluted samples, indicating that the enzymes were stable in the conditions used throughout this work. Incubation times ranged from 15 min to 1 h depending on the activity of the sample.

β-Glucosidase and β-xylosidase assays were based on release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside (Ferchak & Pye, 1983) and *p*-nitrophenyl β-D-xylopyranoside (Sigma) (Biely *et al.*, 1980) respectively. Appropriately diluted samples were incubated with 20 mM-substrate in the presence of 50 mM-potassium phosphate pH 6·5 at 50 °C. The reaction was stopped by addition of 100 mM-NaOH/glycine pH 10·9. Absorbance of the liberated *p*-nitrophenol was measured at 425 nm. Activity units (U) for β-glucosidase and β-xylosidase are μmol *p*-nitrophenol produced min^{-1}.

β-Xylanase was assayed at 50 °C by incubating 1 ml of enzyme solution with 1 ml 100 mM-potassium phosphate pH 8·0 containing 1% (w/v) oat spelt xylan (Sigma). The amount of reducing sugars released was determined by the dinitrosalicylic acid (DNS) method of Miller (1959) with D-xylose as standard. Units of β-xylanase activity (U) are μmol reducing sugars produced min^{-1}.

The activity of endoglucanase was determined at 50 °C by adding 1 ml of enzyme solution to 1 ml 100 mM-potassium phosphate pH 8·0 containing 1% (w/v) carboxymethylcellulose (CMC; Koch-Light 600). The amount of reducing sugars in terms of glucose equivalents was determined either by the DNS procedure mentioned above, or, when high sensitivity was required, by the method of Halliwell & Lovelady (1981). Units of endoglucanase activity (U) are μmol reducing sugars produced min^{-1}.

Growth measurements. During the growth and induction experiments, the production of cell biomass was determined indirectly by estimation of total nitrogen in the solids from the culture using the Kjeldahl procedure (Buchi apparatus). Bacterial protein was calculated as N × 6·25.

A conversion factor between cellular nitrogen and dry weight was determined for cells of the *Streptomyces* sp. grown on CMC and soluble xylan for 48 h. The culture was divided into two parts and filtered on Millipore membranes (0·45 μm). One part served for determination of cellular nitrogen as described above. The other was used for determination of dry weight on pre-weighed membranes, washed twice with water and dried at 105 °C to constant weight (about 48 h). From three independent measurements, 1 mg of cellular nitrogen was equivalent to 13·8 ± 0·15 mg dry weight (mean ± range about the mean). This mean value was used for the estimation of growth yield.

Estimation of cellulose and hemicellulose in cultures. A sample of the culture on straw corresponding to about

30 mg material was filtered on a Millipore 0·45 μm membrane. The residue on the filter was transferred to a vessel containing 90 ml 0·5 M-H_2SO_4, incubated for 12 h at 90 °C and filtered on a Whatman GFA filter.

Total sugars thus released by hemicellulose hydrolysis were estimated in the filtrate by the method of Dubois *et al.* (1956). The value was converted to hemicellulose using a standard obtained with straw hemicellulose prepared according to De Stevens (1957). Cellulose was estimated on the Whatman GFA filters by the procedure of Updegraaf (1969). CMC and xylan were estimated in the culture according to Dubois *et al.* (1956).

Preparation of xylobiose. Xylobiose was obtained by enzymic hydrolysis of oat spelt xylan (Sigma) using crude β-xylanase from the *Streptomyces* sp. The reaction was performed for 12 h at ambient temperature in a dialysis bag. The diffusible fraction was concentrated *in vacuo* at 45 °C and chromatographed on an activated charcoal (Darco G.60-Aldrich) column (2·5 × 35 cm) conditioned with water. The column was eluted with 5% (v/v) ethanol. Carbohydrates in the effluent were detected by the DNS method (Miller, 1959). Fractions containing only xylobiose were identified by high-performance liquid chromatography (HPLC) (Bernier *et al.*, 1983) and pooled. After evaporation, xylobiose was crystallized from aqueous methanol and its identity confirmed by HPLC.

Isoelectrofocusing (IEF). Endoglucanase and β-xylanase activities were detected in culture supernatants by IEF, using the Phast-system of Pharmacia. β-Xylanase activity was detected by the replica gel procedure (Biely *et al.*, 1985*a*, *b*). Endoglucanase activity was detected by a similar procedure using replica gels with 1% (w/v) CMC in 100 mM-potassium phosphate pH 8·0. Congo Red (Theater & Wood, 1982) was used to reveal the undigested substrate. RBR-xylan was purchased from Sigma. Protein bands were revealed by silver staining (Pharmacia Phast-system Manual).

RESULTS

Growth curves and enzyme production by the Streptomyces sp.

Fig. 1 (*a*) shows the growth curve of the strain on 0·2% ball-milled straw. The stationary phase was attained after about 48 h. A similar pattern of growth was observed with other carbon sources and at concentrations ranging from 0·2 to 1% (w/v) (not illustrated). As only about 10% of the nitrogen source was consumed by the time growth ceased in the presence of 1% (w/v) carbon source, these cultures were not nitrogen or carbon limited. Presumably growth stopped because of the production of inhibitory metabolite(s) and/or exhaustion of a micronutrient.

At the stationary phase of cultures with 1% (w/v) CMC or xylan as sole carbon source, the CMC and xylan content was reduced by 69 and 24%, respectively. This corresponded to respective growth yields of 0·48 and 0·49 g microbial dry weight per g CMC and xylan consumed. Some 40% of the hemicellulose but only 5% of the cellulose fraction of ball-milled straw was consumed during the growth phase of the *Streptomyces* sp. (Fig. 1 *a*), corresponding to a growth yield of 0·51 g dry weight per g straw consumed. A decrease in cellular protein was observed after 48 h of growth (Fig. 1 *a*). This probably resulted from cellular lysis: a large proportion of 3-d-old cells no longer retained the Gram stain.

β-Glucosidase and β-xylosidase activities, which were associated with the bacterial cells, rose rapidly during the first 24 h of culture but later declined sharply (Fig. 1 *b*). β-Xylanase and endoglucanase (for which >95% activity was detected in the culture supernatant) behaved differently: endoglucanase activity increased throughout the 3 d of the experiment, while β-xylanase increased to a stable plateau value in 1–2 d (Fig. 1 *b*).

When the *Streptomyces* sp. was grown on a range of carbon sources (results not shown in detail), high activities of xylanolytic enzymes were found only on xylobiose, xylan and straw; endoglucanase was present at high activity only on straw, and surprisingly not (<10% of the specific activity on straw) on CMC, a model cellulose. In contrast, β-glucosidase was expressed at high activities on cellobiose and straw. Low activities (i.e. <10% of the maximal values) were detected on monosaccharide carbon sources (glucose, fructose, mannose, xylose, D-arabinose and rhamnose), while none of the enzyme activities were detected on glycerol as a carbon source.

Expression of enzyme activities in relation to the carbon source was studied in more detail by examining the kinetics of induction–repression, using cells grown on glycerol to inoculate media containing various carbon sources.

Induction and repression pattern

Fig. 2 shows enzyme induction patterns with polymeric substrates and the dimers cellobiose and xylobiose. The enzymes of xylan degradation were apparently induced by straw, xylan or

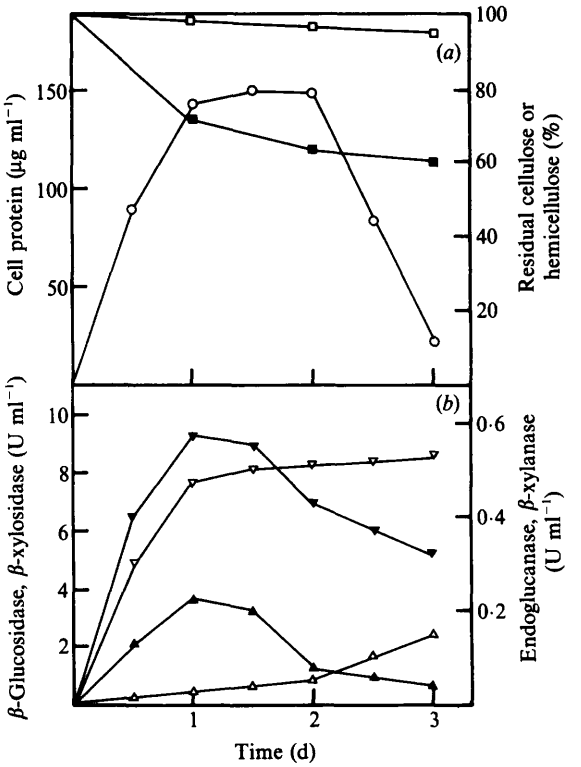

Fig. 1. Growth and enzyme production of *Streptomyces* sp. on 0·2% ball-milled straw. (*a*) Cell protein (○), and percentage of residual cellulose (□) and residual hemicellulose (■). (*b*) Enzyme production: β-glucosidase (▲), endoglucanase (△), β-xylosidase (▼) and β-xylanase (▽). Standard deviations were all within 10% of the mean values presented (three independent assays).

Table 1. *Enzyme induction in Streptomyces sp. and effect of glycerol*

Enzyme activities were induced for 24 h with 0·2% substrate in the absence or (where shown) in the presence of 0·2% glycerol as described in Methods. All enzyme activities are expressed as U (mg protein)$^{-1}$; bacterial protein was determined by Kjeldahl analysis of total N (see Methods). Experiments were done in triplicate; data are mean ± range about the mean. ND, Not detectable.

Substrate	β-Glucosidase	Endoglucanase	β-Xylosidase	β-Xylanase
Glycerol	ND	ND	ND	ND
Straw	8·7 ± 0·4	1·6 ± 0·12	245·0 ± 0·7	5·2 ± 0·2
Straw + glycerol	3·2 ± 0·2	0·63 ± 0·03	19·4 ± 0·6	1·2 ± 0·1
CMC	9·1 ± 0·8	1·4 ± 0·1	3·3 ± 0·3	1·3 ± 0·1
CMC + glycerol	1·3 ± 0·05	0·2 ± 0·03	ND	0·6 ± 0·07
Xylan	1·6 ± 0·07	0·12 ± 0·01	232·0 ± 5	4·7 ± 0·4
Xylan + glycerol	ND	0·06 ± 0·01	61·4 ± 1·3	2·4 ± 0·17
Glucose	ND	0·04 ± 0·01	ND	0·4 ± 0·05
Fructose	0·25 ± 0·02	0·06 ± 0·01	ND	0·29 ± 0·03
Mannose	0·4 ± 0·05	0·12 ± 0·02	ND	0·41 ± 0·04
Xylose	ND	0·15 ± 0·02	ND	0·6 ± 0·04
D-Arabinose	0·02 ± 0·01	0·04 ± 0·01	ND	0·13 ± 0·02
Cellobiose	12·6 ± 0·6	0·10 ± 0·02	0·7 ± 0·03	0·14 ± 0·03
Xylobiose	0·7 ± 0·06	0·10 ± 0·02	280·0 ± 7	5·3 ± 0·1
Kraft lignin	ND	0·87 ± 0·05	1·1 ± 0·02	4·6 ± 0·2
Syringic acid	ND	0·62 ± 0·01	4·1 ± 0·3	9·8 ± 0·3
3,4-Dimethoxycinnamic acid	ND	ND	2·4 ± 0·18	1·8 ± 0·1
3,5-Dimethoxy-4-hydroxycinnamic acid	ND	1·40 ± 0·07	ND	0·15 ± 0·02
Veratric acid	ND	ND	ND	ND
Vanillic acid	ND	ND	ND	ND
Ferulic acid	ND	0·03 ± 0·01	ND	ND

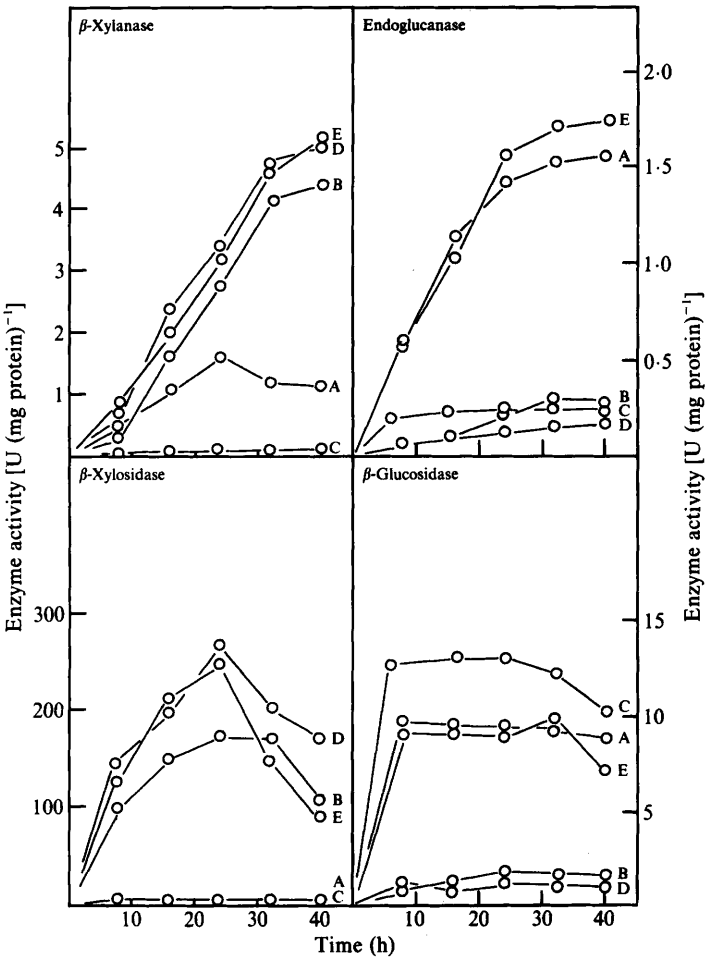

Fig. 2. Enzyme induction in *Streptomyces* sp. on different carbon sources. A, CMC; B, xylan; C, cellobiose; D, xylobiose; E, straw. Standard deviations were all within 10% of the mean values presented (three independent assays).

xylobiose. β-Xylosidase was not induced by CMC, and β-xylanase was poorly induced by this carbon source. When induced on xylan, straw or xylobiose, β-xylosidase attained maximal values 20–30 h after transfer from the glycerol medium and declined thereafter. This pattern was not observed for β-xylanase activity, which attained a plateau level. A decline in β-xylosidase activity was also observed in the growth experiments on straw (Fig. 1*b*).

Endoglucanase and β-glucosidase, enzymes of cellulose degradation, were induced on CMC and straw but only weakly on xylan or xylobiose. Endoglucanase attained a much higher specific activity in induction experiments on CMC than in growing cultures. Cellobiose was a poor inducer for endoglucanase, which was expressed at a slightly higher level than on straw or CMC, but it did induce β-glucosidase (Table 1). Cellotriose and cellotetraose induced β-glucosidase and partially induced endoglucanase (Fig. 3), but did not induce the xylanolytic enzymes (not illustrated). Little or no induction of any of the four enzymes occurred when cells were grown on sugar residues found in lignocellulose: glucose, fructose, mannose, xylose and D-arabinose (Table 1).

Slight differences between the values obtained with the different sugars (Table 1) could be attributed to minimizing catabolite repression. Kraft lignin, chosen as a model lignin, induced

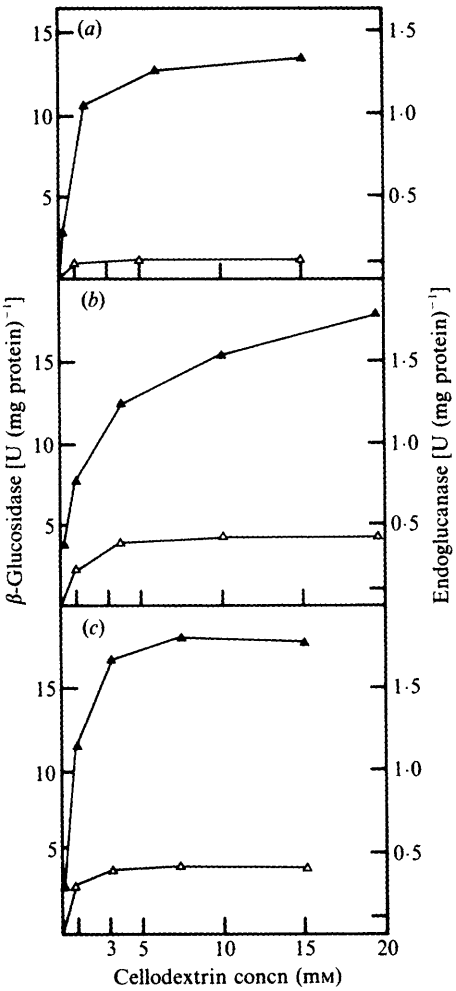

Fig. 3. Effect of cellodextrins on enzyme induction in *Streptomyces* sp. Cells from preculture on glycerol were induced for 24 h in the presence of different concentrations of cellodextrins: (*a*) cellobiose; (*b*) cellotriose; (*c*) cellotetraose. ▲, β-Glucosidase; △, endoglucanase. Standard deviations were all within 10% of the mean values presented (three independent assays).

endoglucanase and β-xylanase but apparently not β-glucosidase or β-xylosidase (Table 1). Products putatively derived from lignin degradation (Flaigl *et al.*, 1975), were also tested as inducers (Table 1). Syringic acid had apparently the same effect as Kraft lignin. 3,4-Dimethoxycinnamic acid partially induced β-xylanase, whereas endoglucanase was the only enzyme induced by 3,5-dimethoxy-4-hydroxycinnamic acid. When glycerol was present in the inducing media (Table 1), the specific enzyme activities attained after 48 h were lower than in the media not supplemented with glycerol. This effect is consistent with a form of carbon catabolite repression effect mediated by glycerol. Furthermore, enzyme induction was prevented by 50 μg chloramphenicol ml^{-1} (not illustrated), indicating that enzyme induction in the *Streptomyces* sp. was likely to be *de novo* synthesis.

Enzyme components in culture filtrates of the Streptomyces sp.

IEF was performed on the supernatants of induction media (Fig. 4). During induction on CMC, only acidic proteins were secreted. Among the three protein bands revealed by silver staining, two (pI 3·55 and 3·75) displayed endoglucanase activity. Cellulase activities were

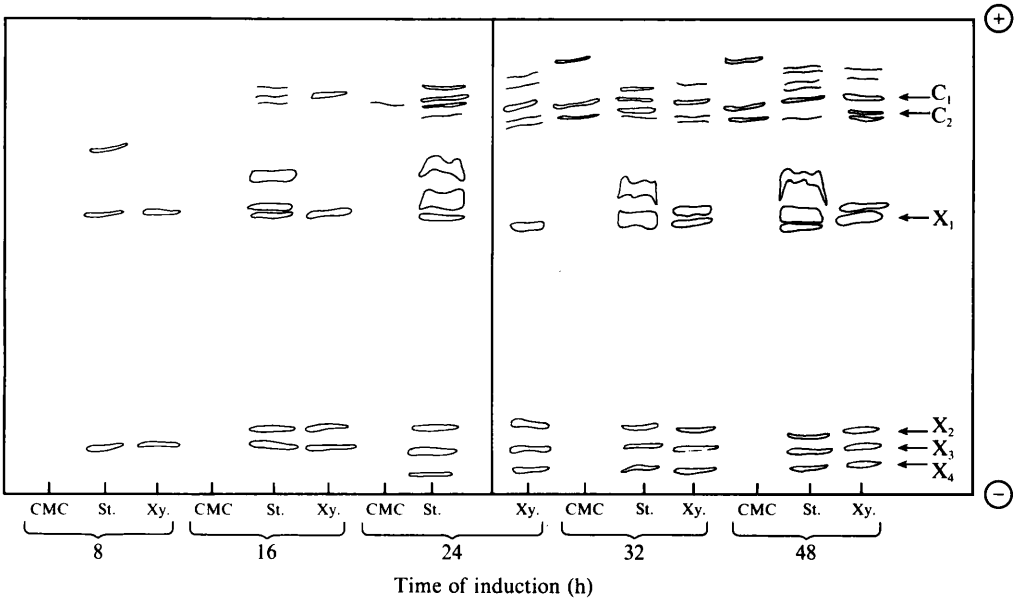

Fig. 4. IEF analysis of the supernatants of induction media. The bands shown are silver-stained proteins. The arrows indicate the different enzyme activities detected on a parallel gel stained for activity: C_1, C_2, endoglucanases; X_1, X_2, X_3, X_4, β-xylanases. Inducers: CMC, straw (St.) and xylan (Xy.).

detectable on straw, and also on xylan. IEF showed that acidic and basic proteins were present in culture fluids of the *Streptomyces* sp. grown on xylan or straw. Four β-xylanase activities (pI 5·2, 8·15, 8·45 and 8·65) were detected (Fig. 4). The acidic component (pI 5·20) was synthesized first, followed successively by the pI 8·45, pI 8·15 and pI 8·65 components.

DISCUSSION

Our results show that at least four electrophoretically distinct β-xylanases and two endoglucanases are produced by this strain of *Streptomyces* sp. The highest specific activities of xylanolytic and cellulolytic enzymes were observed on xylan and CMC respectively. Straw, however, induced the complete set of these enzymes, even though this strain does not appear to degrade the cellulose fraction of straw significantly. Furthermore, some lignin-derived compounds elicited the synthesis of endoglucanase and β-xylanase in this *Streptomyces* sp. This strain is also able to use Kraft lignin and compounds derived from native lignin as sole carbon sources but growth rates and yields are very different (B. Godden, P. Helvenstein & M. Penninckx, unpublished results). Cellotriose and cellotetraose partially induced endoglucanase, and, as fragments derived from cellulose, these molecules may be responsible for the weak induction of endoglucanase observed in batch culture with CMC. High cellulase specific activities in cultures growing on a natural cellulose-containing substrate like straw might result from co-operation between various inducers derived from the components of the lignocellulose complex.

Kluepfel and coworkers (Kluepfel & Ishaque, 1982; Kluepfel *et al.*, 1986) have suggested a possible inducing effect of xylan on endoglucanase in certain *Streptomyces* strains. The rapid degradation of xylan by these strains might, however, explain improved production of endoglucanase in cultures containing xylan. In the present work, where we have expressed the induction data in terms of specific enzyme activities, the specific activity of endoglucanase determined on xylan was only about 27% of the value observed on straw.

In conclusion, it appears that the different component enzymes of lignocellulose degradation

in this strain of *Streptomyces* sp. are subject to distinct controls, with the possibility of some cross-regulation. The low level of cellulase activity against natural cellulose deserves further attention, although it may be that this type of *Streptomyces* must co-operate with other micro-organisms in compost to degrade native straw cellulose completely and efficiently.

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